Identifying Gene Expression Biomarkers to Predict Rodent Cancer Bioassays

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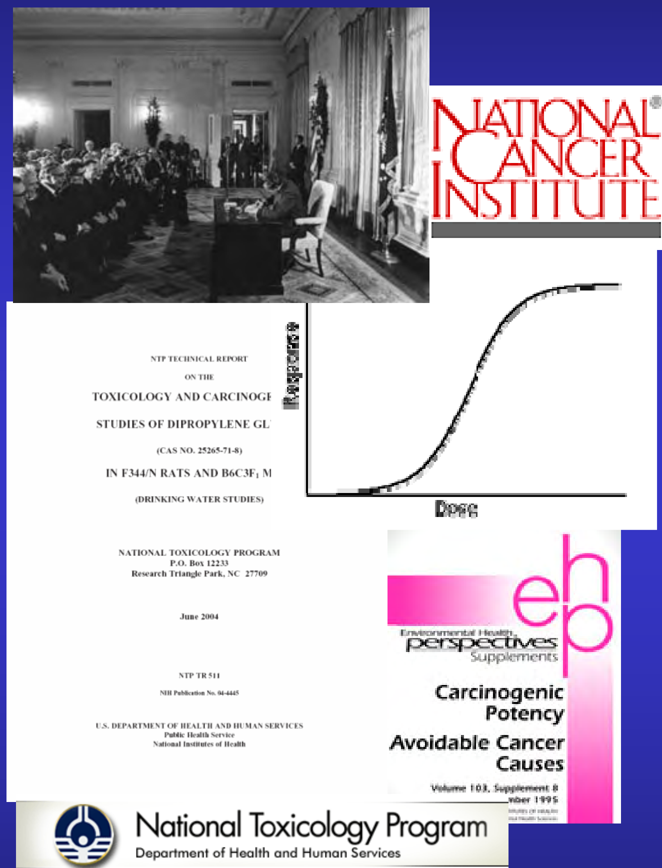
History of the Rodent Cancer Bioassay



- In 1971, President Nixon signed the National Cancer Act. Part of the Act required protecting the public from chemical and physical carcinogenic hazards.
- Originally established as a screen to identify agents that would be further analyzed in human epidemiological studies.
- Today, it has evolved into the <u>primary means</u> to determine the carcinogenic potential of a chemical and generate quantitative information on dose-response behavior in chemical risk assessments.

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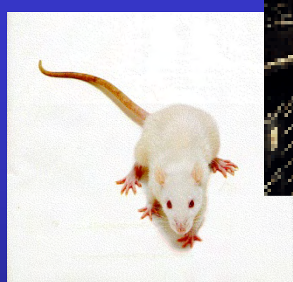
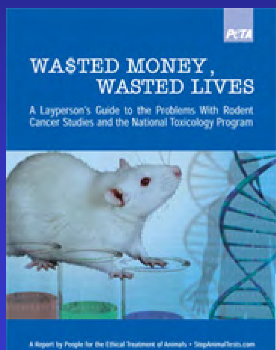
What is a Rodent Cancer Bioassay?



- Involves rats and mice of both sexes (usually B6C3F1 mice and F344/N rats).
- Three dose levels plus vehicle control.
- Highest dose is the maximum tolerated dose.
- Exposures begin at 5 to 6 weeks and last 2 years.
- 50 to 100 animals per dose per sex per species.
- Endpoints include extensive histopathology, clinical chemistry, and physiological measurements.

Why Develop Alternatives to the Rodent Cancer Bioassay?



Tradition Meets High-Tech
Photograph by Peter Essick

At a spa in Maharishi Vedic City, Iowa, John Moore hopes to speed the elimination of PCBs, which are stored in fat for years. Technicians ply him with oils in a purification process based on Indian ayurveda tradition.

Caustic Cuisine
Photograph by Peter Essick

Author David Ewing Duncan cooks breakfast at home. On the menu: PBDEs, phthalates, PCBs, and a side of PFAs.

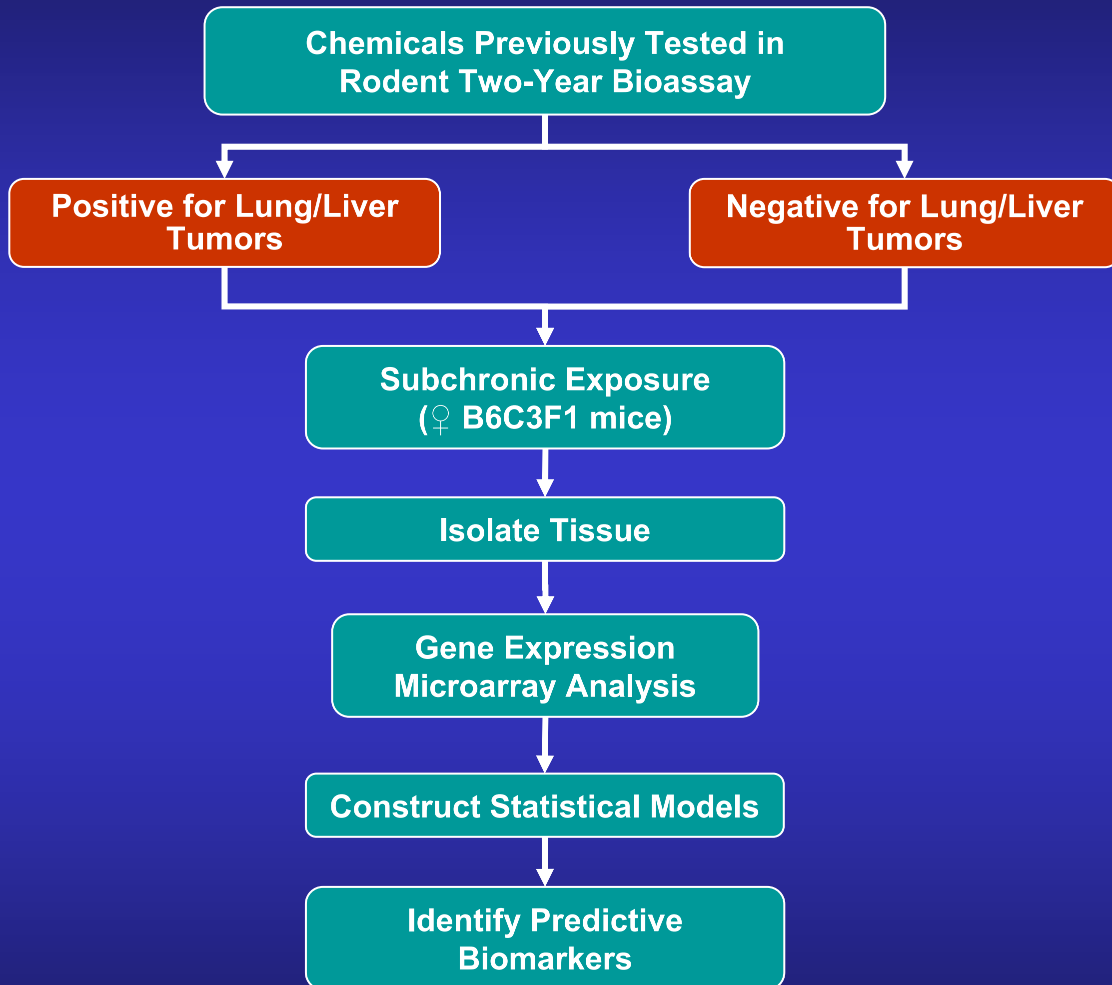
Each year the U.S. Environmental Protection Agency (EPA) reviews an average of 1,700 new compounds that industry is seeking to introduce. Yet the 1976 Toxic Substances Control Act requires that they be tested for any ill effects before approval only if evidence of potential harm exists—which is seldom the case for new chemicals. The agency approves about 90 percent of the new compounds without restrictions. Only a quarter of the 82,000 chemicals in use in the U.S. have ever been tested for toxicity.

- Costs between $2 to $4 million per chemical (1996 dollars).
- Typically performed late in the developmental pipeline where a positive response can delay/prohibit product release.
- 1,468 chemicals have been tested in a rodent cancer bioassay (CPD, 2005).
- 90,000 chemicals on the EPA TSCA inventory and ~9,000 chemicals used in quantities >10,000 lbs.
- "The lack of knowledge about the impact of many chemicals on human health and the environment is a cause for concern" (*EC REACH white paper*)

Central Question

Can existing two-year bioassay data generated by the National Toxicology Program be used to identify short-term biomarkers that are predictive of tumor formation in a two-year rodent bioassay?

Experimental Design



Thomas *et al.* *Toxicol Sci.* 96:40, 2007
Thomas *et al.* *Toxicol Sci.* 97:55, 2007

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Chemicals in the Study

Chemical	Abbreviation	NTP No.	Route	Dose in Study
N-Methylolacrylamide	MACR	352	Gavage[b]	50 mg/kg
1,2-Dibromoethane	DBET	86	Gavage[a]	62 mg/kg
Benzene	BENZ	289	Gavage[a]	100 mg/kg
Coumarin	COUM	422	Gavage[a]	200 mg/kg
Benzofuran	BFUR	370	Gavage[a]	240 mg/kg
Tris(2,3-dibromopropyl)phosphate	TDPP	76	Feed	1,000 ppm
2,2-Bis(bromomethyl)-1,3-propanediol	BBMP	452	Feed	1,250 ppm
1,5-Naphthalenediamine	NAPD	143	Feed	2,000 ppm
1-Amino-2,4-dibromoanthraquinone	ADBQ	383	Feed	20,000 ppm
Iodoform	IODO	110	Gavage[a]	93 mg/kg
Diazinon	DIAZ	137	Feed	200 ppm
2-Chloromethylpyridine hydrochloride	CMPH	178	Gavage[b]	250 mg/kg
Tetrafluoroethylene	TFEL	450	Inhalation	1,250 ppm
N-(1-naphthyl)ethylenediamine dihydrochloride	NEDD	168	Feed	3,000 ppm (2,000 ppm)[c]
Trichlorofluoromethane	TCFM	106	Gavage[a]	3,925 mg/kg
Pentachloronitrobenzene	PCNB	61	Feed	8,187 ppm
4-Nitroanthranilic acid	NAAC	109	Feed	10,000 ppm
Malathion	MALA	24	Feed	16,000 ppm[d] (14,800 ppm)
Tetrafluoroethane	TFEA	---[e]	Inhalation	50,000 ppm
Water	WCON		Gavage[b]	
Corn oil	CCON		Gavage[a]	
Feed	FCON		Feed	
Air	ACON		Inhalation	

[a]Gavage exposure with a corn oil vehicle (5 ml/kg).

[b]Gavage exposure with a deionized water vehicle (5 ml/kg).

[c]The initial dose of 3,000 ppm was reduced to 2,000 ppm in week 2 of the study due to taste aversion and weight loss. The 2,000 ppm dose is the same as the low dose in the original bioassay.

[d]Due to signs of toxicity, the 16,000 ppm dose was reduced to 0 ppm on day 9 for a period of 2 days. The dose was raised to 8,000 ppm for a period of 9 days and returned to 16,000 ppm for the remainder of the study. The time weighted average dose was 14,800 ppm.

[e]Chemical not evaluated by the NTP. Bioassay performed by Alexander *et al. Hum. Exp. Toxicol.* 14:706, 1995.

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Structural Diversity Among Chemical Treatments

Tanimoto Similarity Coefficients Among the 19 Chemicals Used in the Study

	IODO	DIAZ	CMPH	TFEL	NEDD	TCFM	PCNB	NAAC	MALA	TFEA	MACR	DBET	BENZ	COUM	BFUR	TDPP	BBMP	NAPD	ADBQ
IODO	1.000	0.024	0.019	0.091	0.030	0.091	0.025	0.021	0.017	0.125	0.045	0.103	0.094	0.021	0.033	0.036	0.054	0.051	0.018
DIAZ		1.000	0.214	0.039	0.144	0.034	0.170	0.209	0.241	0.036	0.099	0.023	0.037	0.234	0.116	0.193	0.060	0.104	0.218
CMPH			1.000	0.024	0.187	0.076	0.216	0.203	0.127	0.049	0.071	0.051	0.105	0.162	0.130	0.117	0.060	0.192	0.179
TFEL				1.000	0.064	0.278	0.063	0.049	0.034	0.394	0.120	0.105	0.098	0.035	0.071	0.055	0.113	0.081	0.032
NEDD					1.000	0.064	0.268	0.247	0.104	0.064	0.157	0.051	0.175	0.148	0.258	0.067	0.055	0.508	0.223
TCFM						1.000	0.074	0.045	0.034	0.314	0.073	0.105	0.071	0.031	0.046	0.063	0.095	0.057	0.035
PCNB							1.000	0.394	0.150	0.058	0.104	0.024	0.112	0.151	0.157	0.101	0.057	0.267	0.207
NAAC								1.000	0.202	0.058	0.147	0.042	0.095	0.276	0.158	0.110	0.099	0.233	0.337
MALA									1.000	0.049	0.108	0.031	0.034	0.238	0.083	0.179	0.094	0.058	0.216
TFEA										1.000	0.096	0.167	0.071	0.035	0.046	0.070	0.131	0.081	0.035
MACR											1.000	0.100	0.041	0.130	0.072	0.123	0.178	0.103	0.116
DBET												1.000	0.079	0.031	0.031	0.146	0.327	0.047	0.038
BENZ													1.000	0.079	0.195	0.020	0.046	0.314	0.066
COUM														1.000	0.175	0.118	0.095	0.102	0.288
BFUR															1.000	0.090	0.074	0.317	0.173
TDPP																1.000	0.294	0.047	0.097
BBMP																	1.000	0.055	0.067
NAPD																		1.000	0.174
ADBQ																			1.000

For Tanimoto similarity coefficients → 1 = identical, 0 = no similarity.

Average similarity among chemicals is 0.116.

Average similarity among all NTP chemicals was 0.155.

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Genotoxic Diversity Among Chemical Treatments

Chemical	Ames Assay Results
N-Methylolacrylamide	-
1,2-Dibromoethane	+
Benzene	-
Coumarin	+
Benzofuran	-
Tris(2,3-dibromopropyl)phosphate	
2,2-Bis(bromomethyl)-1,3-propanediol	+, -, +
1,5-Naphthalenediamine	+
1-Amino-2,4-dibromoanthraquinone	+
Iodoform	+,+
Diazinon	-
2-Chloromethylpyridine hydrochloride	+
Tetrafluoroethylene	
N-(1-naphthyl)ethylenediamine dihydrochloride	+
Trichlorofluoromethane	-,-
Pentachloronitrobenzene	-
4-Nitroanthranilic acid	+, +
Tetrafluoroethane	
Malathion	-

Lung Tumor Incidence for Chemical Treatments

	Incidence of Alveolar/Bronchiolar Adenomas or Carcinomas in Female B6C3F1 Mice					
Chemical	Control	Low	Mid	High	Relative Dose in Present Study	Classification
N-Methylolacrylamide	6/50	8/50		13/49*	High	Lung Carc
1,2-Dibromoethane	0/20	11/43*		6/46	Low	Lung Carc
Benzene	4/49	5/42	10/50	13/49*	High	Lung Carc
Coumarin	2/51	5/49	7/49	27/51*	High	Lung Carc
Benzofuran	2/50	9/48*		14/47*	High	Lung Carc
Tris(2,3-dibromopropyl)phosphate	4/55	9/50		17/50*	High	Lung Carc
2,2-Bis(bromomethyl)-1,3-propanediol	5/52	5/50	15/51*	19/50*	High	Lung Carc
1,5-Naphthalenediamine	0/49	10/48*		5/46*	High	Lung Carc
1-Amino-2,4-dibromoanthraquinone	4/50	17/50*		15/49*	High	Lung Carc
Iodoform	1/20	1/49		0/45	High	Non Lung Carc
Diazinon	1/23	1/46		2/49	High	Non Lung Carc
2-Chloromethylpyridine hydrochloride	1/19	1/49		3/48	High	Non Lung Carc
Tetrafluoroethylene	6/48	1/48	8/47	4/47	High	Non Lung Carc
N-(1-naphthyl) ethylenediamine dihydrochloride	0/49	2/48		1/31	Low	Non Lung Carc
Trichlorofluoromethane	2/19	0/50		1/47	High	Non Lung Carc
Pentachloronitrobenzene	0/20	0/23		1/20	High	Non Lung Carc
4-Nitroanthranilic acid	1/45	5/41		1/48	High	Non Lung Carc
Tetrafluoroethane	3/120	4/60	4/60	2/60	High	Non Lung Carc
Malathion	0/10	0/49		0/47	High	Non Lung Carc

Liver Tumor Incidence for Chemical Treatments

Chemical	Incidence of Hepatocellular Adenomas or Carcinomas in Female B6C3F1 Mice: Control	Low	Mid	High	Relative Dose in Present Study	Classification
N-Methylolacrylamide	6/50	7/50		17/49*	High	Liver Carc
Benzofuran	4/50	25/48*		22/47*	High	Liver Carc
Tris(2,3-dibromopropyl)phosphate	11/54	23/50*		35/49*	High	Liver Carc
1,5-Naphthalenediamine	1/46	28/49*		27/46*	High	Liver Carc
1-Amino-2,4-dibromoanthraquinone	6/50	46/50*		50/50*	High	Liver Carc
Tetrafluoroethylene	17/48	33/48*	29/47*	28/47*	High	Liver Carc
Benzene	4/49	12/44*	13/50*	7/49	High	Liver Carc*
Coumarin	8/50	27/49*	31//51*	13/50	High	Liver Carc*
1,2-Dibromoethane	0/20	1/44		0/47	Low	Non Liver Carc
2,2-Bis(bromomethyl)-1,3-propanediol	20/51	19/50	9/50	18/49	High	Non Liver Carc
Iodoform	1/20	1/49		0/45	High	Non Liver Carc
Diazinon	2/23	0/47		3/49	High	Non Liver Carc
2-Chloromethylpyridine hydrochloride	0/20	1/49		0/49	High	Non Liver Carc
N-(1-naphthyl) ethylenediamine dihydrochloride	1/46	1/48		1/30	Low	Non Liver Carc
Trichlorofluoromethane	1/19	4/50		2/49	High	Non Liver Carc
Pentachloronitrobenzene	0/20	0/14		3/20	High	Non Liver Carc
4-Nitroanthranilic acid	4/45	0/41		1/47	High	Non Liver Carc
Tetrafluoroethane	12/120	1/60	8/60	4/60	High	Non Liver Carc
Malathion	0/10	0/49		2/47	High	Non Liver Carc

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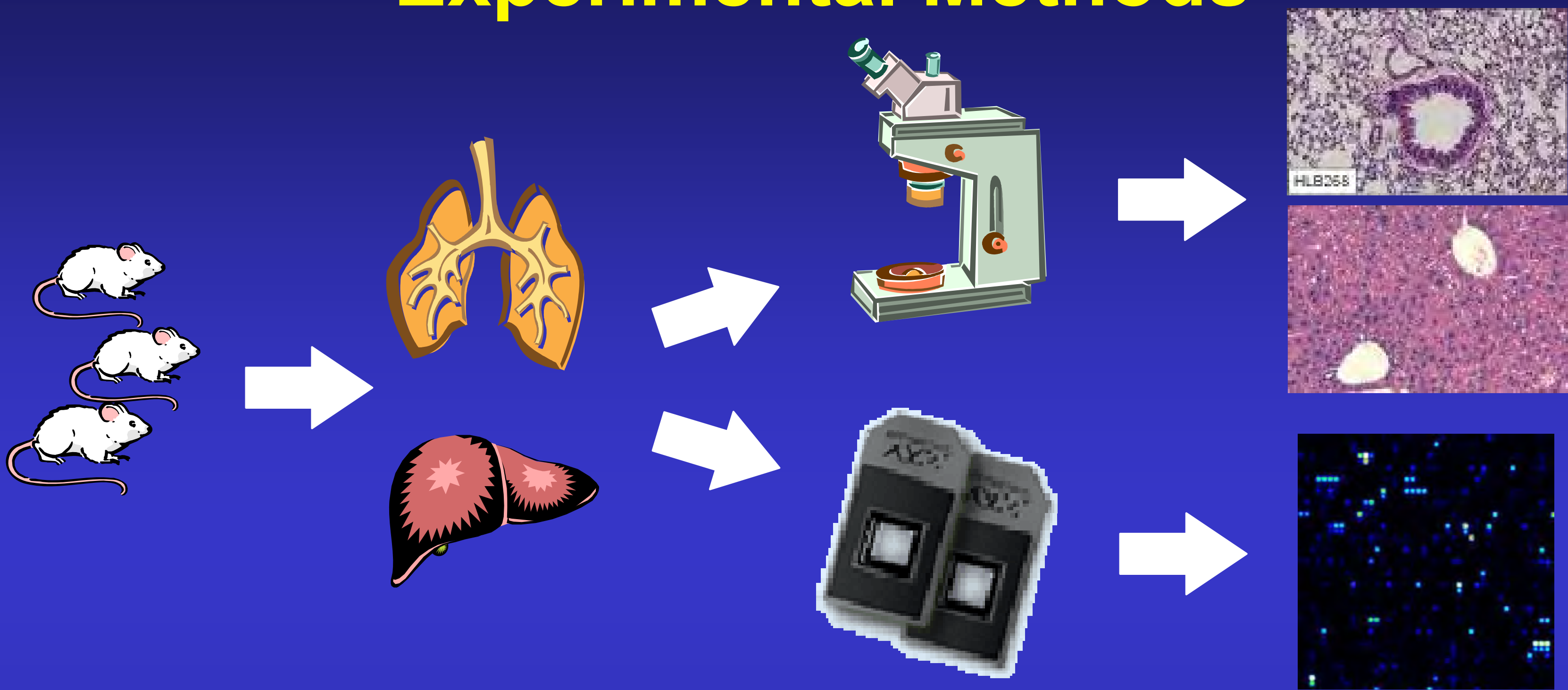
Experimental Methods



- 5-6 week old female B6C3F1 mice (10 mice per treatment group) were exposed for 13 weeks.
- Histopathology on left lung lobe and isolate RNA from right lobes
- Histopathology on left liver lobe and isolate RNA from right, caudate, and median lobes
- Microarray analysis on 3 - 4 animals per treatment group using Affymetrix 430 2.0 arrays.

Histopathology Results

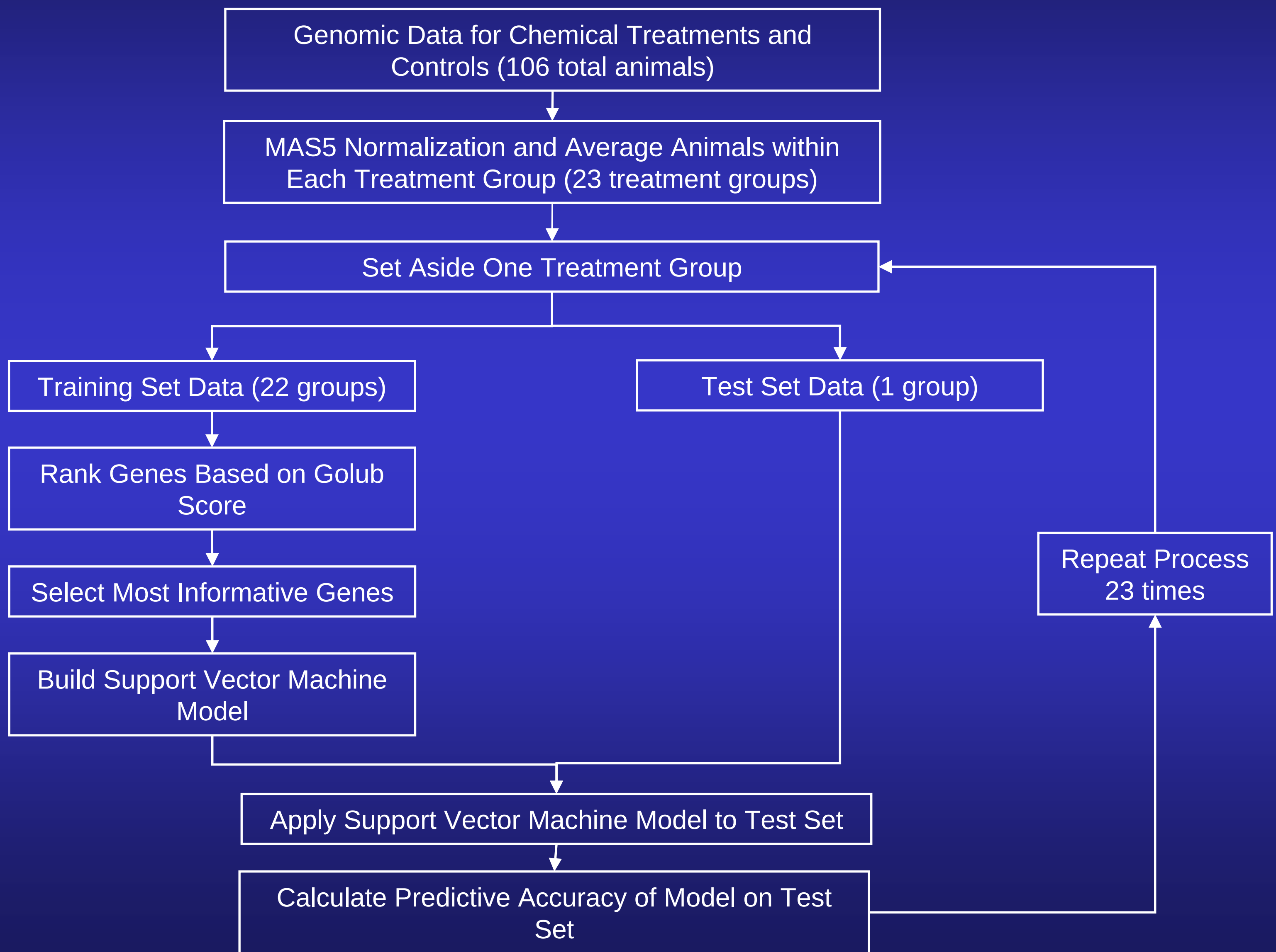
Lung:

- Gross histological changes observed in only one chemical.
- 1,5-Naphthalenediamine produced karyomegaly and karyorrhexis in brochiolar epithelial cells and occasional peribronchiolar infiltration by neutrophils and mononuclear cells.

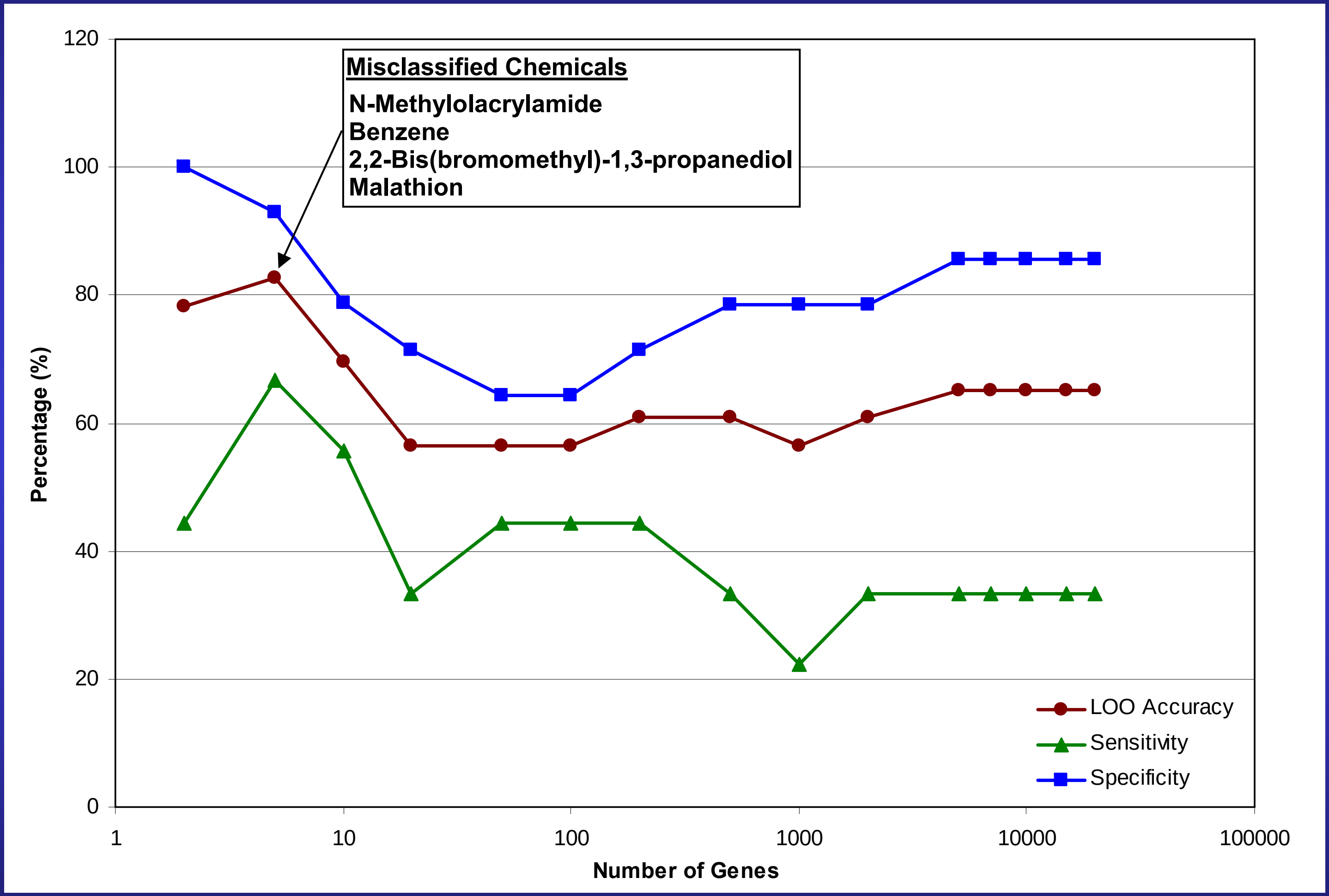
Liver:

- Gross histological changes observed in only one chemical.
- Benzofuran produced minor single cell necrosis.

Identifying Important Biomarkers and Building Classification Model



Predictive Accuracy of the Lung Gene Expression Biomarkers



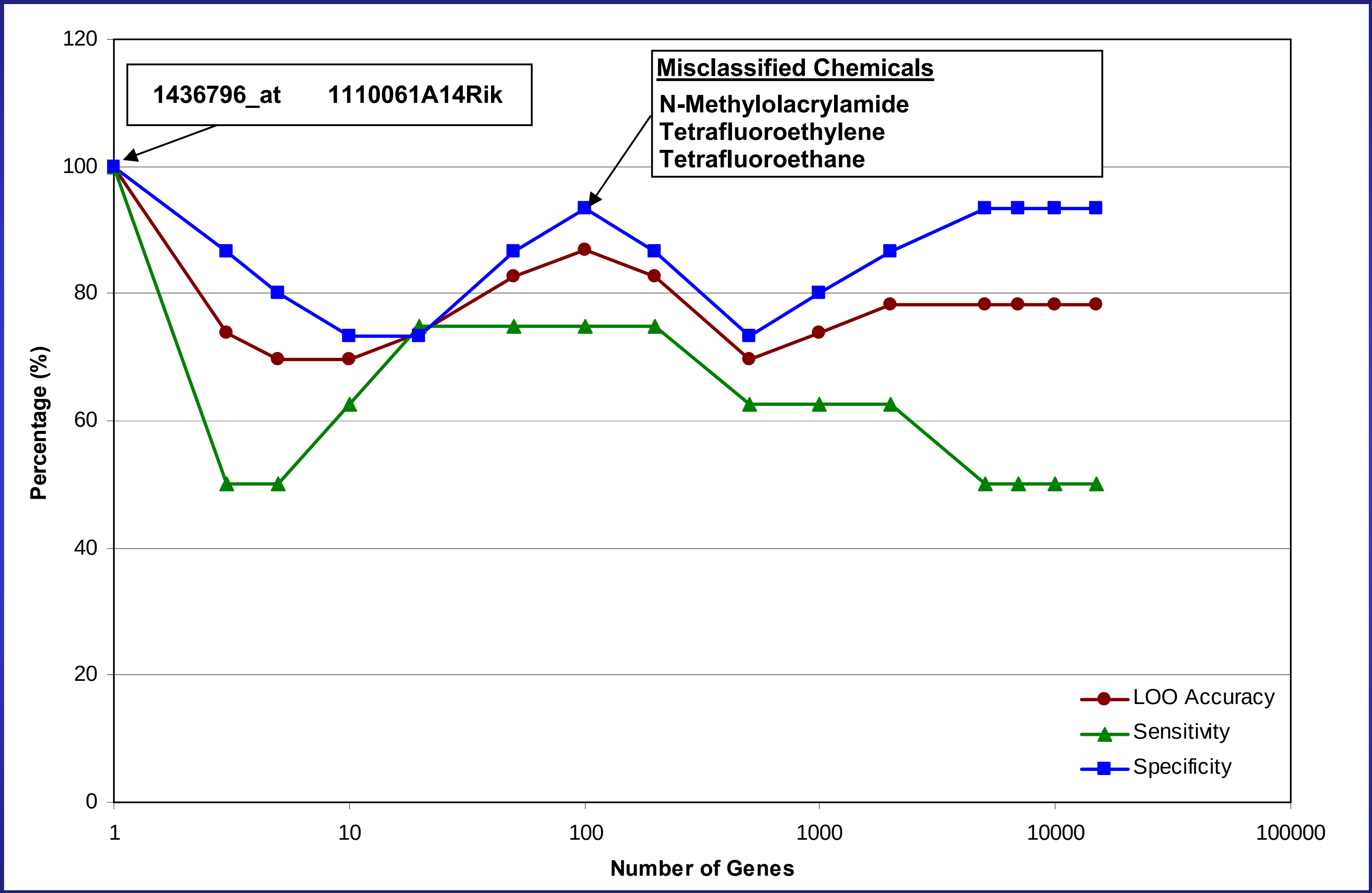
***SVM model, linear kernel, LOO cross-validation**

List of Top Lung Gene Expression Biomarkers

Top gene expression biomarkers in the lung that discriminate between carcinogenic and noncarcinogenic treatments based on the Golub algorithm

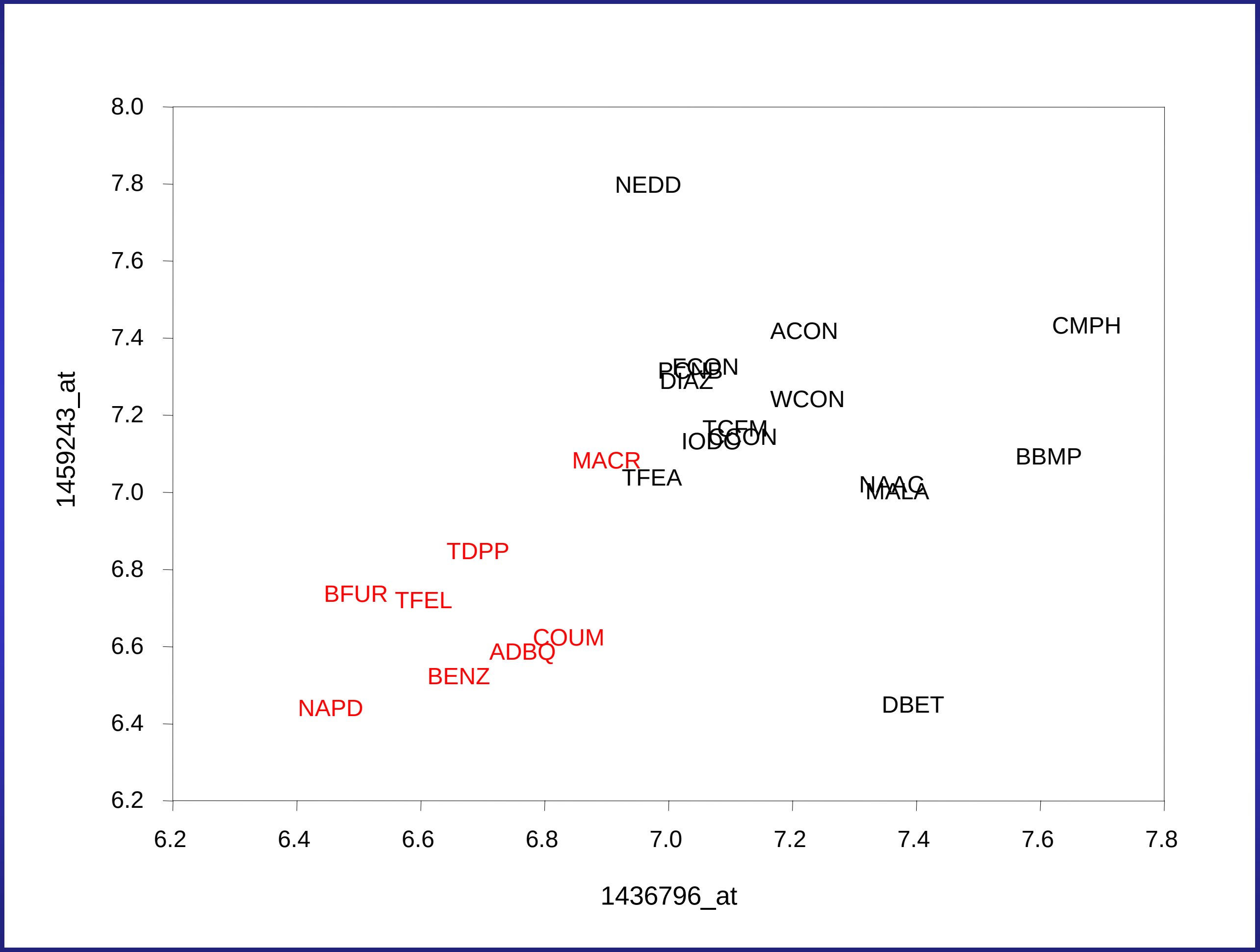
Affymetrix ID	Transcript ID	Gene Symbol	Gene Description	Golub Score	Expression Among Carcinogens
1425627_x_at	Mm.2011.2	Gstm1	Glutathione S-transferase, mu 1	1.171685	Increased
1444139_at	Mm.205420.1	Ddit4l	DNA-damage-inducible transcript 4-like	1.087507	Increased
1435647_at	Mm.200976.1	Ikbkg	Inhibitor of kappaB kinase gamma	0.990865	Increased
1425626_at	Mm.2011.2	Gstm1	Glutathione S-transferase, mu 1	0.96369	Increased
1449486_at	Mm.22720.1	Ces1	Carboxylesterase 1	0.885322	Increased

Predictive Accuracy of the Liver Gene Expression Biomarkers



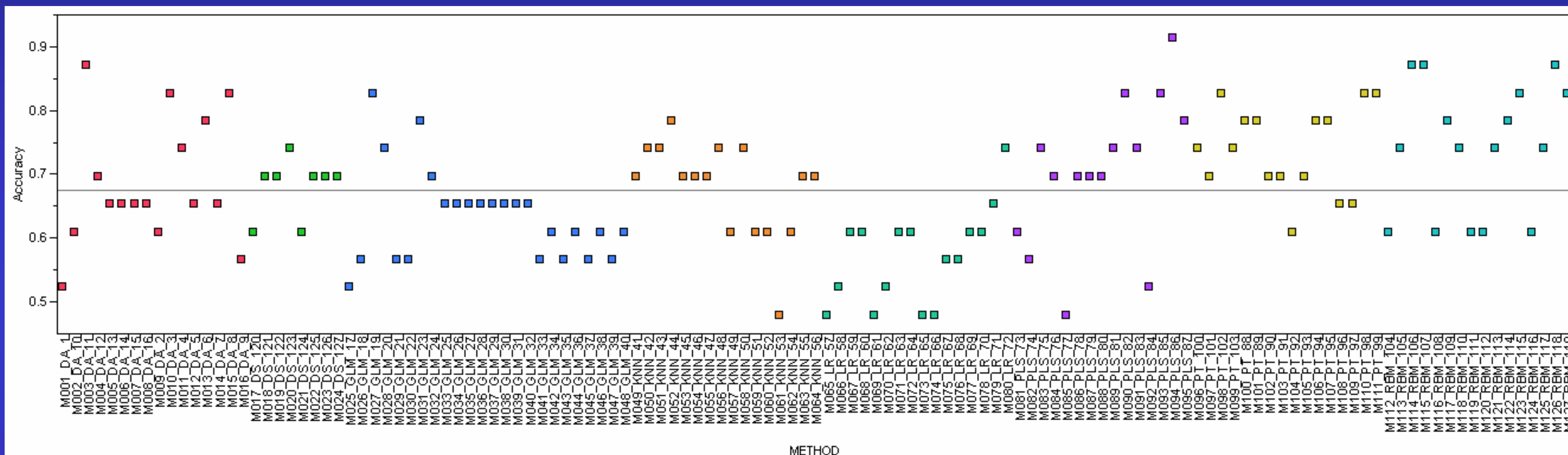
***SVM model, linear kernel, LOO cross-validation**

Most Discriminating Liver Gene Expression Biomarkers



Predictive Accuracy of the Liver Gene Expression Biomarkers

Differences in LOOCV Accuracy Using Various Classification Algorithms



Discriminant Analysis | Distance Scoring | General Linear Models | K-Nearest Neighbors | Logistic Regression | Partial Least Squares | Partition Trees | Support Vector Machines

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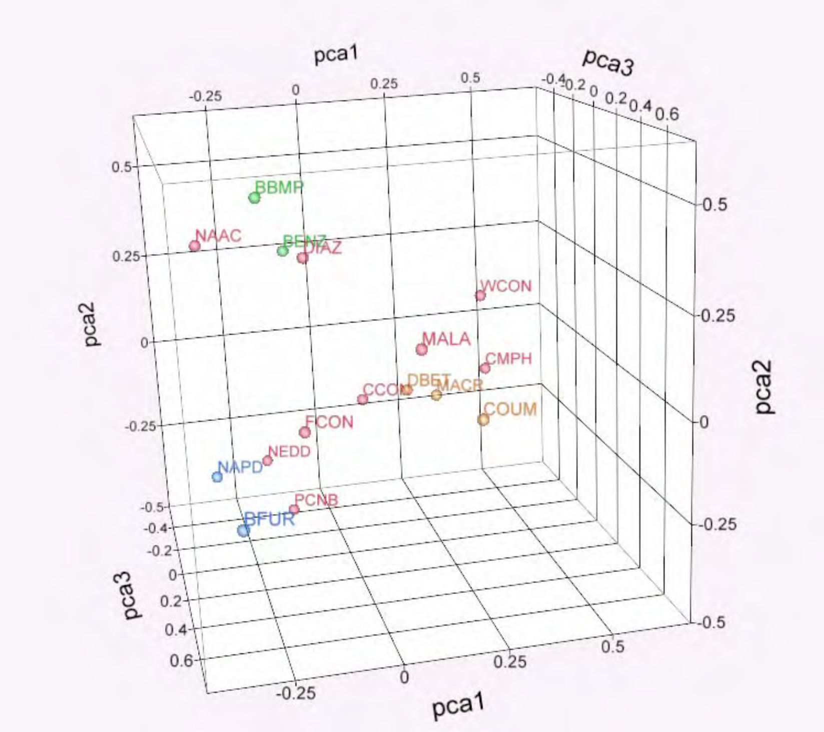
Conclusions

- Gene expression biomarkers collected following a subchronic exposure can predict increased tumor incidence in a two-year bioassay with reasonable accuracy.
- The carcinogenic chemicals used in the study were intentionally chosen to be diverse in chemical structure, genotoxicity categories, and potential modes-of-action.
- The approach appears promising for application across multiple target organs.

Path Forward

- Explore potential “mechanistic separation” among carcinogens.
- Build a set of screening biomarkers for four key tissues that can identify ~64% of all positive NTP chemicals.
- Investigate other potential non-tissue specific biomarkers.
- Investigate time course and dose-response behavior of biomarkers.
- Explore microdissection techniques to reduce potential “tumor incidence detection limit”

Path Forward



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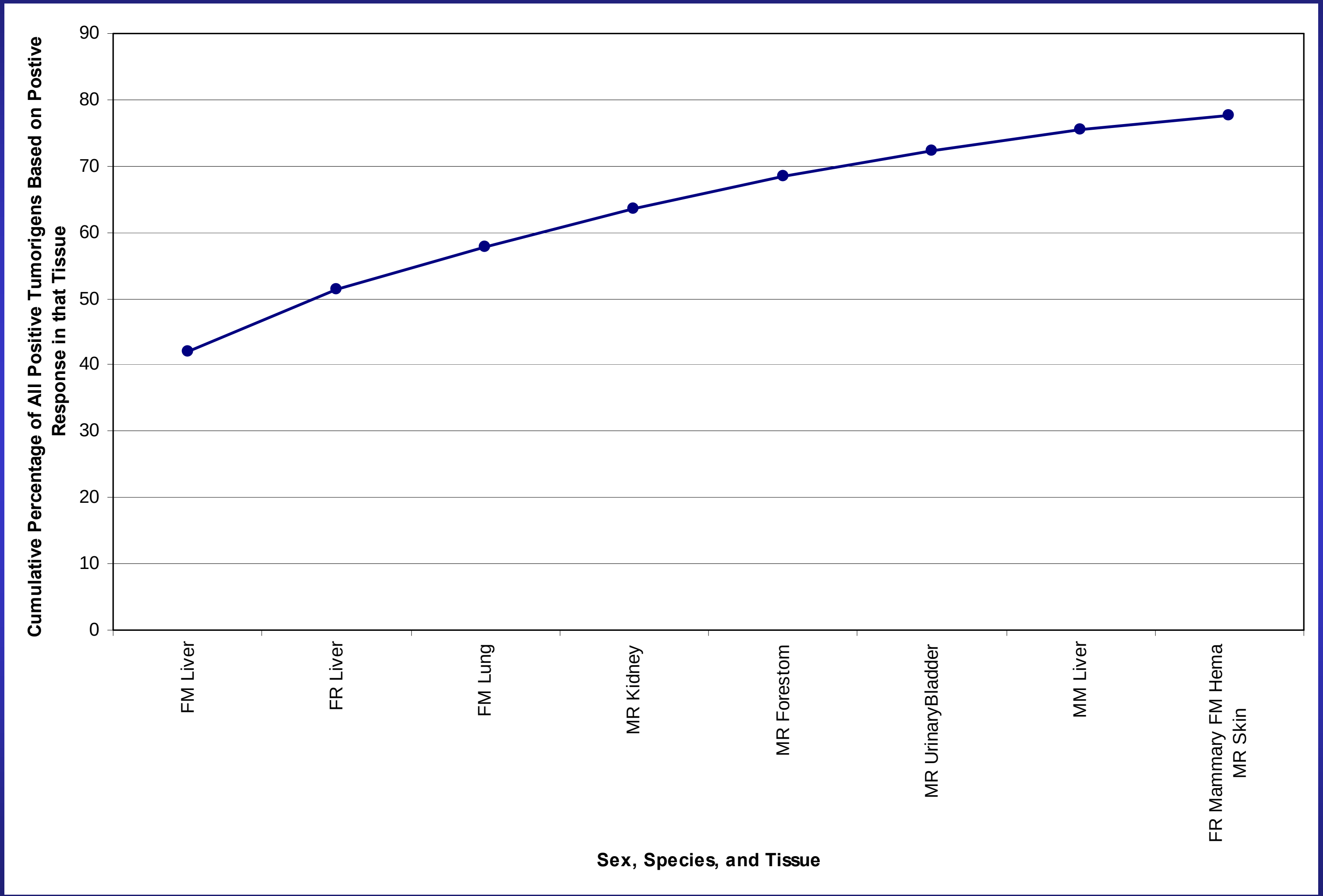

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